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13. ABSTRACT *(Maximum 200 words)*
Rats were created that are transgenic for the neu proto-oncogene in order to establish a rat model of neu-mediated mammary tumorigenesis. The mouse mammary tumor virus promoter drives the expression of neu. Three transgenic lines have been generated and one of these lines has been maintained homozygous for the transgene. To date, all three lines show a low incidence of spontaneous mammary tumorigenesis. The vast majority of tumors that have arisen in the oldest line appear after one year of age and are fibroadenomas. Examination of mammary gland whole mounts from both sexes revealed no differences in gross ductal morphology between non-transgenic and transgenic rats of any line. The overexpression of neu within the transgenic mammary gland has not yet been confirmed. Initial attempts to address this question using immunohistochemistry failed due to an unexpected high level of endogenous neu expression in non-transgenic rats. In addition to the generation of transgenic rats, a retroviral expression vector was constructed that utilizes green fluorescent protein as the selectable marker. Members of the EGFR-family of tyrosine kinase growth factor receptors have been cloned into this retroviral vector and concentrated retroviral stocks have been prepared for each construct.

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INTRODUCTION

Tyrosine kinase growth factor receptors make up an important class of proteins involved in the growth and differentiation of eukaryotic cells. The epidermal growth factor receptor (EGFR) became the first member of the type I family of tyrosine kinase growth factor receptors. Since then, three other proteins have been added to this family; HER2/erbB2/neu, HER3/erbB3, and HER4/erbB4. (For the remainder of this proposal, the erbB nomenclature will be used to name the proteins). All four members of this family are approximately 180 kD single-chain transmembrane spanning proteins composed of an extracellular ligand binding domain, a cytoplasmic tyrosine kinase domain, and a cytoplasmic C-terminus. erbB2/neu, erbB3, and erbB4 share significant homology to EGFR. In particular, the tyrosine kinase domains are highly conserved among the four members while the C-terminus shows the lowest degree of homology (reviewed in 1).

Aberrant overexpression of the erbB receptors has been reported to be associated with several human malignancies (2-5). In particular, overexpression of EGFR and erbB2/neu is found in a large percentage of human breast cancers. This overexpression is generally correlated with a poor patient prognosis (6). Additionally, erbB3 overexpression has also been associated with some human breast cancers, but this relationship for erbB3 is not as straightforward as for EGFR and erbB2/neu in terms of patient prognosis (7).

Although overexpression of erbB2/neu in human breast cancer is found in a large percentage of cases, the exact role that erbB2/neu plays in the etiology of human breast cancer is unknown. In order to investigate the role of erbB2/neu overexpression in mammary gland tumorigenesis, Muller and colleagues generated mice transgenic for the neu proto-oncogene (neu N) under the control of the mouse mammary tumor virus (MMTV) promoter (8). These mice developed mammary tumors at a high incidence with a latency of 8-9 months and the tumors were associated with overexpression of neu. Additionally, these tumors showed increased neu tyrosine kinase activity. However, follow up studies revealed that the majority of tumors in these transgenic mice contained somatic mutations in the neu transgene (9). These mutations resulted in the constitutive dimerization of neu, leading to the elevated neu tyrosine kinase activity (10). In human breast tumors, however, activating mutations in erbB2/neu have not been detected. Therefore, these studies with the transgenic mice cannot be exactly correlated with the role of neu N in human breast tumorigenesis. Recently published reports also indicate that the susceptibility of the MMTV-neu N mouse to mammary tumor formation can be influenced by the genetic background. When MMTV-neu N mice were bred into another strain, the tumor latency was dramatically increased (11). These results, combined with Muller's observation of activating mutations in the transgene, indicate that other factors play important roles in the mammary tumorigenesis of MMTV-neu N transgenic mice.

In addition to the mouse, the rat is a commonly used animal model in breast cancer research. The rat model has a number of differences with the mouse model. Unlike mice, rats and humans do not have a viral origin for mammary tumorigenesis. Like humans, the majority of rat mammary tumors are hormone responsive. Additionally, rat mammary tumors more closely resemble human mammary tumors in terms of histological characteristics. Because of these differences, we became interested in knowing if rats transgenic for MMTV-neu N would display the same susceptibility to mammary tumorigenesis due to the overexpression of neu as seen in the transgenic mice.

Of particular interest to us is whether neu N can cooperate with other members of the EGFR family of tyrosine kinase growth factor receptors. It is a well-established fact that members of this family can form heterodimers with each other (reviewed in 1). Specifically, erbB2/neu can form heterodimers with each of the other three members of the family and appears to be their preferred heterodimer partner (12). erbB2/neu is an orphan receptor, with no known ligand. However, the ability of erbB2/neu to form heterodimers allows for signaling by various ligands when the ligand is bound to the heterodimer partner (reviewed in 1). In particular, a erbB2/neu heterodimer with EGFR is responsive to ligands of the epidermal growth factor/transforming growth factor α (EGF/TGFα) family which bind to EGFR. Likewise, erbB2/neu heterodimers with erbB3 or erbB4 are responsive to ligands of the heregulin/neu differentiation factor (NDF) family. This complex network of heterodimer and homodimer formation among members of the family potentially regulate numerous growth and differentiation signal transduction pathways.

Certain heterodimer combinations result in an increased transforming potential in NIH 3T3 cells. Specifically, erbB2/neu was transforming when coexpressed with erbB3 or EGFR (13). In mice doubly transgenic for neu N and TGFα, there is a synergistic interaction between the two genes that lead to a decreased tumor latency. The authors suggest that this synergy arises from a EGFR:neu N interaction (14).

Based on all of the available evidence, it seems plausible that neu N in our transgenic rats would be capable of forming heterodimers with the other members of the protein family if they were co-overexpressed. It is tempting to speculate that the co-overexpression of some or all of the other family members will lead to a synergistic interaction for mammary tumorigenesis. Investigating the potential cooperativity of neu N and the other family members in our transgenic rat model could help to delineate the role that erbB2/neu overexpression plays in the etiology and progression of human breast cancer.

BODY

METHODS

Preparation of Transgenic DNA for Microinjection

90 µg of MMTV-neu N plasmid DNA (8) was digested with SphI and EcoRI. The digested DNA was resolved on a 1% SeaKem GTG agarose gel (FMC Bioproducts). The 9 kb transgene was electroeluted into 8 M ammonium acetate, then the purified DNA was precipitated and resuspended in TE buffer. The DNA was then further purified by passing through an Elutip-d column (Schleicher & Schuell) and the eluate was precipitated and resuspended in TLE buffer (10 mM Tris pH 7.5/0.1 mM EDTA). Following resuspension, the DNA was microdialyzed against TLE using type V_M 0.05 µm filters (Millipore). The dialyzed DNA was then used for microinjections.

Generation of MMTV-neu N Transgenic Rats

The technical aspects of generating transgenic rats is reviewed in (15). Briefly, 30-day old Sprague-Dawley (SD) female rats were superovulated with follicle-stimulating hormone and luteinizing hormone then mated overnight. The next day, single-cell embryos were flushed from the oviducts and cultured in M16 media until the pronuclei became visible. The embryos were then microinjected in the large pronucleus with linearized, transgene DNA. Following a 1-2 hr culture in M16 media, the microinjected embryos were reimplanted into pseudopregnant SD female recipients. Approximately 12-18 injected embryos were implanted into each recipient's oviduct.

Isolation of Genomic DNA

0.5 cm long tail clips were cut from rats and minced in 0.6 ml of genomic lysis solution (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 100 mM EDTA, 1% SDS) with 4 µl of proteinase K stock solution (14 mg/ml, Boehringer Mannheim). The minced tissue was digested overnight at 55^0. The next day, the samples were cooled to room temperature then spun in a microcentrifuge at 14,000 rpm for 5 min to pellet undigested material. The supernatant was transferred to a clean tube containing 200 µl of Protein Precipitation Solution (Gentra Systems) and vortexed vigorously at high speed for 20 sec. To precipitate proteins, the samples were then spun at 14,000 rpm for 3 min in a microcentrifuge. The supernatant was poured into a clean tube containing 0.6 ml isopropanol and the samples were then gently inverted continuously for 1 min. After a 30 min incubation at -20^0, the samples were spun at 14,000 rpm for 1 min in a microcentrifuge to precipitate DNA. The supernatant was discarded and the DNA pellet was washed with 0.5 ml of 70% ethanol by inverting the tube several times followed by a 1 min spin at 14,000 rpm in a microcentrifuge. The supernatant was discarded and the DNA was allowed to airdry for 15-30 min. The DNA was then resuspended overnight in 200 μl of dH_2O.

PCR Transgenic Screening

To screen for the transgene, 100-500 ng of genomic DNA was subjected to PCR using primers complementary to the SV40 region of the transgene. The primers used were: forward primer (Tag 3) 5'-ACTCCACACAGGCATAGAGTGTCTGC-3' and reverse primer (Tag 4) 5'-AGGACACAGAGGAGCTTCCTGGGGAT-3' (Genosys). Reaction conditions were:

a) initial denaturing at 95^0 for 5 min
b) denaturing at 92^0 for 45 sec
c) annealing at 60^0 for 45 sec
d) extension at 72^0 for 1 min

35 cycles were done of b-d, then a final extension at 72^0 for 5 min, followed by soaking at 23^0. The PCR reaction was analyzed on a 0.8% agarose gel. For some experiments, independent PCR reactions were run under the same conditions with primers for ras as a test of DNA quality. The primers used were: forward primer (5' HRAS) 5'-TGGCTAGGGCCTGGCTAAGT-3' and reverse primer (3' HRAS) 5'-CTGGTCCCGCATGGCACTAT-3' (Genosys).

Generation of Homozygote Transgenic Rats

Heterozygote transgenic males were mated with heterozygote transgenic females. The progeny were screened by PCR and those rats found to be transgenic were test bred with non-transgenic rats. The progeny of the test breedings were screened by PCR. The transgenic rats being tested were scored as homozygote if 100% of 10 or more of the test progeny were transgenic. Homozygote transgenic lines were established and maintained by mating homozygote transgenic males with homozygote transgenic females.

Whole Mounts of Rat Mammary Glands

Mammary glands were removed and spread onto glass slides and allowed to sit dry for 5 min. The glands were then placed overnight in 70% ethanol. The next day, the glands were fixed in 100% ethanol: glacial acetic acid (3: 1) for 1 hr. After fixing, the glands were washed in 70% ethanol for 15 min, 50% ethanol for 5 min, and dH_20 for 5 min. The glands were then stained for 2-4 days in alum carmine solution [per 500 ml of dH_20: 2.5 g alum potassium sulfate, 1.0 g carmine; boiled for 20 min and filtered]. After staining, the glands were successively defatted in 70%, 95%, and 100% ethanol for 30 min each. The glands were then placed in xylene overnight and transferred to mineral oil for long-term storage.

Immunohistochemistry

Tissues were fixed in 3% buffered formalin, embedded in paraffin, and sectioned onto coated slides. All incubation temperatures are at room temperature unless otherwise specified. The sections were deparaffinized in three changes of xylene for 5 min each. The sections were then rehydrated with 100% ethanol (2 x 10 min), 95% ethanol (2 x 10 min), and water (5 min) then treated with 0.5% hydrogen peroxide to quench endogenous peroxidase activity. After 5 min washes each in water and PBS, the sections were treated for 5 min with trypsin at 1 mg/ml (Sigma, #T-7168) followed by washes in PBS (3 x 5 min). The sections were next blocked with PBS/10% normal goat serum for 1 hr. Blocking solution was aspirated off and primary antibody was then bound overnight at 4°. Primary antibodies were diluted to 0.5 μg/ml in PBS/1% bovine serum albumin (BSA) (Sigma, #A-7906) with 5% normal goat serum. The primary antibodies used were rabbit anti-neu polyclonal IgG (Santa Cruz Biotechnology, #sc-284) and normal rabbit IgG (Santa Cruz Biotechnology, #sc-2027). After washes in PBS (3 x 5 min), goat anti-rabbit biotinylated IgG (Vector Laboratories) was bound for 30 min. Biotinylated secondary antibody was diluted 1:200 in PBS/1% BSA with 1.5% normal goat serum. After washes in PBS (3 x 5 min), ABC reagent (Vectastain Elite ABC Kit, Vector Laboratories) was applied for 30 min and then the sections were washed again in PBS (3 x 5 min). The sections were then incubated in PBS/1% Triton X-100 for 30 sec, rinsed in water, and stained with DAB (Sigma, #D-4293) for 2 min. After a rinse in water, the sections were counterstained with Mayer's hematoxylin solution (Sigma, #MHS-16) for 30 sec, rinsed in water, and successively dehydrated with 95% ethanol, 100% ethanol, and xylene (2 x 10 sec for each). Finally, the sections were mounted with Permount (Fisher).

Construction of Retroviral Expression Vectors

pLSG is a derivative of the vector pLCG.2, which in turn was derived from the vector pLCG.1. To construct pLCG.1, pS65T GFP-C1 (Clontech) [GFP=green fluorescent protein] was digested with BamHI and BglII to destroy the provided multiple cloning site. Following this digestion, pS65T GFP-C1 was re-ligated. The ligated product destroyed both the BamHI and BglII sites, but created a new BstYI site. This modified pS65T GFP-C1 was used to transform the E. coli strain SCS110 (Stratagene). Plasmid DNA was isolated from transformants and digested with NsiI and BclI to remove a 1.4 kb cassette containing the CMV promoter and S65T GFP coding region. This cassette was ligated into pGEM-7Zf (Promega) digested with NsiI and BamHI and used to transform the E. coli strain DH5α (Gibco BRL). All subsequent transformations were also done in DH5α. The CMV-S65T GFP cassette was then removed from pGEM-7Zf using NsiI and HindIII and ligated into pSP73 (Promega) digested with PstI and HindIII. Meanwhile, pEGFP-1 (Clontech) [EGFP=enhanced green fluorescent protein] was digested with BamHI and

NotI to remove the EGFP coding region. EGFP was ligated into pCEP4 (Invitrogen) digested with BamHI and NotI. S65T GFP was removed from pSP73 by digestion with AgeI and HindIII. EGFP was removed from pCEP4 by digestion with AgeI and HindIII. EGFP was then linked up by ligation to the CMV promoter already cloned into pSP73, thereby replacing S65T GFP. The retroviral expression vector pJR (16) was digested with SalI and HindIII to remove the SV40 promoter-neo-pBR322 ori cassette. The CMV-EGFP cassette was removed from pSP73 by digestion with SalI and HindIII and ligated to pJR to create a new retroviral expression vector, pLCG.1. To construct pLCG.2, oligos 5'-GATCCAGATCTGGGCCCGTTAACCCTAGGG-3' and 5'-TCGACCCTAGGGTTAACGGGCCCAGATCTG-3' (University of Wisconsin-Madison Biotechnology Center) were annealed to form overhanging BamHI and SalI ends. The annealed oligo pair was then ligated to pLCG.1 digested with BamHI and SalI to create pLCG.2. To construct pLSG, PCR was used to amplify the SV40 promoter from the vector pLXSN (17). The forward primer was 5′-CCGGAATTCGTTAAGTCGACCTCGAGGATCCGGC-3′ and the reverse primer was 5′-TCCGCCTCGACCACCGGTTGCAGCCCAAGC-3′ (University of Wisconsin-Madison Biotechnology Center). These primers created new SalI and AgeI sites that flank the SV40 promoter sequence. The 400 bp amplified SV40 promoter was digested with SalI and AgeI and then ligated to pLCG.2 that had previously been digested with SalI and AgeI to remove the CMV promoter. This created the retroviral expression vector pLSG. A schematic diagram of pLSG is depicted in figure 2.

To construct the vector pLSG/EGFR, a 4.1 kb cassette containing the human EGFR cDNA was released from the vector pLXSN/EGFR (18) by digestion with XhoI and ligated to pLSG digested with SalI. To construct pLSG/erbB4, a 4.6 kb cassette containing the human erbB4 cDNA was released from the vector pLXSN/erbB4 (18) by digestion with SalI and ligated to pLSG digested with SalI. To construct pLSG/neu N, the plasmid MMTV-neu N was digested with HindIII and the 5′ overhanging end was filled in with Klenow polymerase. Next, MMTV-neu N was digested with SalI to release a 4.5 kb cassette containing the rat neu N cDNA. This neu N cassette was ligated to pLSG digested with HpaI and SalI. To construct pLSG/neu T (point-mutated, activated neu), pJR/neu T (19) was digested with EcoRI. A 6.2 kb fragment containing the rat neu T cDNA and the SV40 promoter was isolated and treated with Klenow polymerase to fill in both overhanging ends. The 6.2 kb fragment was then digested with SalI to release the 4.6 kb neu T cDNA and this was subsequently ligated to pLSG digested with HpaI and SalI.

Generation of pLSG-based Retroviruses

pLSG or derivatives were transfected into the ecotropic retroviral packaging producer line Ψ-CRE (20) using lipofectAMINE (Gibco BRL) and the manufacturer's recommended protocol. Two days after transfection, conditioned media containing ecotropic recombinant virus was collected and used to infect the amphotropic retroviral packaging producer line PA317 (21).

Two days later, the infected PA317 were passed through a FACStar^{+} cell sorter (Becton Dickinson) equipped with an argon ion laser tuned to 488 nm and ran at 50mwatts. GFP fluorescence data was collected on a logarithmic amplified scale (four log decades) and forward, side scatter data was collected on a linear scale. Forward scatter width data on a linear scale was used to eliminate cellular aggregates. GFP^{+} cells were sorted and then placed into complete media. The sorted cells were cultured for a few days then were trypsinized and counted with a hemacytometer. 15-30 cells were placed into each of several 100 mm culture dishes and cultured for 10 days to allow for the growth of individual clones. 20-30 clones were then harvested and expanded. Conditioned media from each clone was collected and screened for viral titers. The clones producing the highest amounts of virus were expanded and conditioned media was collected. The virus in the conditioned media was concentrated by ultracentrifugation through a sucrose cushion as described (16).

Titering pLSG-based Retroviruses

Concentrated viral samples were serially diluted with tenfold dilutions in media and 1 ml of the diluted viral samples was used to infect NIH 3T3 cells. Two days after infection, the infected cells were passed through a FACScan flow cytometer (Becton Dickinson) at a flow rate of 1 μl/sec. The instrument parameters were set with linear amplification of forward and side scatter (1024 channels) and logarithmic amplification of GFP fluorescence (four log decades). Single cells were gated and a defined number of gated cells were collected. For screening viral producer clones, 10,000 cells were collected for each sample. This number was increased to 50,000 cells for titering concentrated viral samples. The software used for data analysis was Cell Quest (Becton Dickinson). In order to determine the best viral producer clone, the percentages of GFP^{+} cells found in each sample were compared. To calculate the titer of concentrated viral samples, the following formula was used:

a) $\frac{\text{50,000 collected cells}}{\text{collection time (sec)}} \times \frac{\text{sec}}{1\ \mu\text{l}} \times \frac{1000\ \mu\text{l}}{\text{ml}} = \text{cellular density in } \frac{\text{cells}}{\text{ml}}$

b) $\frac{\text{cells}}{\text{ml}} \times \text{volume of cell suspension (ml)} = \text{total cell number}$

c) total cell number x % of GFP^{+} cells = total number of GFP^{+} cells

d) $\text{total number of GFP}^{+}\text{ cells} \times \text{cellular replication factor (=0.5)} \times \text{viral dilution factor} = \frac{\text{GFU}}{\text{ml}}$

GFU = Green Fluorescent Units.

RESULTS

Generation of MMTV-neu N Transgenic Rats

To create transgenic rats, the transgene MMTV-neu N was microinjected into SD pronuclei. All microinjections were done at the University of Wisconsin-Madison Biotechnology Center. 32 potential founder rats were screened by PCR for the presence of the transgene using genomic DNA prepared from tail clips. As a control for DNA quality, all samples were also screened by PCR for ras. Of the 32 rats screened, two were positive for the transgene as indicated by a 350 bp PCR product. These transgene positive rats were identified as 6490 (male) and 6500 (male). All 32 rats were positive for ras as indicated by a 500 bp PCR product. Founders 6490 and 6500 were mated with female SD and the offspring were screened for the transgene. Founder 6490 sired 16 female F_1 and 15 male F_1. 43.8% of the females and 66.7% of the males were transgenic. Founder 6500 sired 12 female F_1 and 15 male F_1, of which 50.0% and 33.3% were transgenic, respectively. Transgenic line 43ll had been created previously.

Generation of Homozygote 4311 MMTV-neu N Transgenic Rats

In order to establish homozygote 4311 transgenic rats, heterozygote transgenic rats were mated together and the resulting offspring were then test bred against non-transgenic rats. A total of 12 transgenic males and 12 transgenic females were test bred. Of the tested males, 3 were determined to be homozygote by virtue of passing the transgene to 100% of the test progeny. The respective numbers of offspring from each homozygote male were 17, 18, and 25. Of the tested females, 2 were determined to be homozygote by using the same criteria. The respective numbers of offspring from each homozygote female were 11 and 20. A homozygote line of 4311 transgenics has been maintained by breeding homozygote rats together.

Analysis of Rat Mammary Whole Mounts in Transgenic Rats

To determine if the presence of the neu N transgene alters the gross morphology of the mammary gland, whole mounts of mammary glands were examined in male rats, virgin female rats and day 10 pregnant rats for each transgenic line. Females were 3-4 months old at the time of tissue collection and males were 5 months old. The transgenics were compared against non-transgenic SD rats. Whole mounts were stained as described under Methods. The whole mounts were qualitatively analyzed for the degree of ductal branching and the size of the alveolar buds. For both males and females (virgin and pregnant), there was no difference between non-transgenic rats and transgenic rats of any line in terms of the gross mammary gland morphology (data not shown). For both virgin and pregnant females, 2 rats were examined for non-transgenic and line 6490, and 1 rat was examined for lines 4311 and 6500. For males, 6 rats were examined for non-transgenic and 4 rats were examined for lines 6490 and 4311. At the present time, whole mounts of males for line 6500 have not been analyzed.

Expression of neu in the Virgin Mammary Gland

In order to qualitatively assess the expression of neu within the virgin mammary gland, tissues were collected from non-transgenic and transgenic females of all three lines and subjected to immunohistochemistry as described under Methods. The females were 3-4 months old at the time of tissue collection. 2 rats were examined for non-transgenic and line 6490 and 1 rat was examined for lines 4311 and 6500. In addition to these samples, the following were also examined: a spontaneous mammary carcinoma that arose in a 4 month old 6490 virgin female and a spontaneous mammary fibroadenoma and normal mammary gland from a 1.5 year old virgin non-transgenic female. For all samples, serial sections were blotted with either a rabbit anti-neu polyclonal IgG or normal rabbit IgG as a negative control. The normal rabbit IgG did not result in DAB staining for any of the samples. In contrast, anti-neu blotting resulted in intense DAB staining for all samples examined. There was no qualitative differences between non-transgenic and transgenic mammary glands. Staining occurred in both the epithelial and stromal elements of the glands. The staining was cytoplasmic, and did not appear to be localized to the membrane. Figure 1 depicts the results from a 3 month old non-transgenic and these are representative of the other samples analyzed.

Generation of Recombinant Retroviruses

Concentrated stocks of recombinant retroviruses have been prepared for EGFR, neu N, neu T, and erbB4 as described under Methods. Figure 3 shows the results from a titering assay of concentrated LSG/neu T virus. This figure is representative of the titering results for all other concentrated viruses.

DISCUSSION

Phenotypes of the MMTV-neu N Transgenic Rats

Lines 6490 and 6500 are newly generated within the last year and it is too early to definitively conclude the phenotype of either line. Founder males 6490 and 6500 are 13 months and 10 months old, respectively. Both are without any abnormal phenotype. For line 6490, there has been 1 virgin female that had 1 mammary carcinoma upon necropsy at 4 months of age. At the present time, there are 7 6490 uniparous females at 9 months of age and 3 6490 virgin females at 8 months old; all tumor free. Presently, there are 4 6500 virgin females at 6 months old and all are tumor free. Line 4311 has existed the longest time in our laboratory and females of this line are generally tumor free through the first year of life. The majority of tumors that have been obtained from this line have been fibroadenomas, although a small number of carcinomas have also arisen. Males of this line have gone out to 1.5 years of age to date with no abnormal phenotype. At this early date, it appears that lines 6490 and 6500 mimic line 4311 in showing a low incidence of mammary tumor formation. These results contrast those of the MMTV-neu N mice created by Muller and colleagues, which show a high incidence of mammary carcinomas at 8-9 months of age.

A key unanswered question is the expression level of neu within the transgenic mammary gland. We have initially begun to answer this question using immunohistochemistry. However, non-transgenic rats displayed a high degree of endogenous neu expression, making it impossible to determine if the transgenic rats overexpress neu. This was unexpected, as numerous papers have reported that neu is not expressed in the normal mammary gland of the mouse or the human. Because of this unexpected finding, we question the validity of the results obtained. It might be possible that the particular anti-neu antibody used in our experiment was cross-reacting with some other common antigen. To address this issue, we are currently performing immunohistochemistry with a different commercial source of an anti-neu antibody. We also have plans to investigate the expression levels of neu by using nuclease protection assays. This combination of methodologies will hopefully determine if the transgenic rats overexpress neu. Until this question is answered, it will not be possible to adequately compare our transgenic rats with Muller's transgenic mice.

Although we do not know the expression levels of neu in our transgenics, we can state that the gross ductal morphology of the transgenic mammary gland does not differ from non-transgenic rats. This is true for both male and female rats. Surprisingly, all male SD rats showed a well-developed mammary gland, with an extensive ductal network. There was little difference between virgin female and male mammary glands.

Finally, in order to maximize the transgene dosage, we have generated and maintained homozygote rats for line 4311. We are currently in the process of generating homozygotes for lines 6490 and 6500.

<u>Generation of Recombinant Retroviruses</u>

We have generated concentrated viral stocks for recombinant retroviruses expressing EGFR, neu N, neu T, and erbB4. All of these genes were cloned into a retroviral vector, pLSG, created in this laboratory by the author. Sequencing of the 5′ ends of the genes was done to confirm that all inserts were cloned in the sense orientation. erbB3 was also cloned into pLSG, but subsequent sequence analysis revealed that erbB3 was mistakenly cloned in the antisense orientation. Therefore, a recombinant retrovirus for erbB3 has not been generated yet, but this will be done in the near future. pLSG was used for recombinant vector construction instead of pLCG. This was because of some preliminary evidence that indicated the CMV promoter of pLCG might be interfering with expression from the LTR. Our extensive experience with the vector pJR allows us to conclude that the SV40 promoter does not seem to interfere with LTR expression. Therefore, pLSG was used here since it uses the SV40 promoter to drive GFP expression instead of the CMV promoter.

pLSG utilizes GFP as a selection marker instead of neomycinphosphotransferase (neo), which has been the standard selection marker used in virtually all retroviral vectors. We chose to utilize GFP as the selection marker because of its usefulness in allowing the infected cells to be sorted by flow cytometry. This attribute should prove useful in future experiments, where the sorting of infected primary rat mammary epithelial cells will be done. Neo is so commonly used because it provides resistance to the cytotoxic drug geneticin, also known as G418. Retrovirally-infected cells grown in the presence of G418 survive, while uninfected cells die. Thus, the infected cells form clones which can be quantitated. This results in retroviral titers being expressed as colony forming units/ml (CFU/ml). Because pLSG lacks neo, titers had to be expressed in another arbitrary unit. We chose to express LSG-viral titers as GFU/ml, which reflects the number of GFP^+ cells as determined by flow cytometry. To our knowledge, there are two descriptions in the literature of GFP being used to titer retroviral vectors (22, 23). The authors report viral titers to be on the order of 10^6 infectious particles/ml for unconcentrated viral solutions. For the viral stocks that we generated for this report, we achieved titers ranging from 3×10^7 - 2×10^8 GFU/ml. Our viral titers are one to two orders of magnitude higher because of an additional concentration step. Importantly, our titer unit of GFU/ml corresponds well with GFP titer units reported by other laboratories. In addition to these two reports, there are several other retroviral vectors expressing GFP to have been described in the literature (24, 25). These vectors, however, use drug resistance genes to titer the viruses.

The inclusion of GFP in our retroviral vector does not appear to adversely affect transformation of NIH 3T3 cells from the oncogenes cloned under the control of the viral LTR. LSG/neu T PA317 viral-producer clones were readily transformed by day 10 post-infection as evidenced by focal growth. These transformation properties are the same as seen previously with JR/neu T viral-producer clones, which express neo instead of GFP. Although LSG/EGFR viral-producer clones were not transformed under normal growth conditions, the inclusion of human EGF at 10 ng/ml in the growth media did transform the clones by the 9th day of treatment.

At the present time, we do not know if the inclusion of GFP in our retroviral vector will inhibit oncogene-mediated tumorigenesis *in vivo* , although we do not expect this to be the case. We also do not know how a viral titer expressed as GFU/ml relates to a titer expressed as CFU/ml. We have extensive experience in using JR/neu T to induce mammary tumors and we know the dose response of the titer to total tumor burden. In order to address these questions, we set up an experiment to compare the dose responses of JR/neu T and LSG/neu T at two different, numerically equal titers. At this point, the experiment is at a very early stage and there is not any data to report.

In the first annual report, we reported that pantropic packaged retroviral vectors were 45-fold more efficient at infecting rat mammary epithelial cells *in vivo* than amphotropic packaged vectors. This number was derived by counting the number of blue cells *in situ* /mammary gland following X-gal histochemistry of JR/gal infused glands. Since then, we have attempted to duplicate these results in a more quantitative experiment. Wistar-Furth rats were infused with both Pan-LCG.1 and Amp-LCG.1 vectors. LCG.1 only differs from LSG in the use of the CMV promoter to drive GFP expression, instead of the SV40 promoter. At both days 3 and day 10 post-infusion, primary rat mammary epithelial cells were isolated and analyzed by flow cytometry to determine the percentage of GFP^+ cells present in the gland. Unexpectedly, Pan-LCG.1 and Amp-LCG.1 yielded the same percentage of GFP^+ cells. We do not know the reason for the very large discrepancy in the results obtained by the two different methods nor do we know which is the "real" result. Because the earlier result of a 45-fold difference was not supported by the flow cytometry quantitation, we decided to use amphotropic instead of pantropic vectors for our experiments. The reasons for this are two fold. First, our laboratory has much more experience in using amphotropic vectors. Secondly, pantropic vectors are considerably more labor intensive to make than amphotropic vectors.

CONCLUSION

In this report, we describe the generation of two additional lines of MMTV-neu N transgenic rats. This brings to three the total number of lines so far generated. Line 4311 was the first line generated. These rats are generally tumor-free throughout their first year of life. The majority of spontaneous mammary tumors that do arise in this line are fibroadenomas, with but a small number of carcinomas occurring. The newly created lines, 6490 and 6500, have only a few females of each to date. The oldest females of lines 6490 and 6500 are 9 months and 6 months old, respectively. So far, there has been only one mammary tumor in line 6490 and no mammary tumors have arisen in line 6500. Although it is too early to definitively conclude, these two new lines of MMTV-neu N transgenic rats appear to share the same phenotype of line 4311; namely a low incidence of spontaneous mammary tumor formation. Additionally, we established a line of homozygote 4311 transgenics.

Attempts have been made to determine if the transgenic rats overexpress neu within the mammary gland by using immunohistochemistry. This attempt was unsuccessful, because non-transgenic rats were found to have a high degree of endogenous neu expression and there was no qualitative differences compared to transgenic rats. This result was unexpected, as the normal human and mouse mammary gland has not been reported to express appreciable levels of neu. Therefore, the results obtained here may be due to problems with the particular antibody used. We are planning on trying these experiments again using a different antibody as well as using nuclease protection assays to quantify the transgene message. Even if there is overexpression of neu, the presence of the transgene does not alter the gross ductal morphology of the transgenic mammary gland compared to non-transgenic rats.

In addition to the generation of transgenic rats, we have constructed a retroviral vector that uses GFP as the selectable marker. Members of the EGFR-family of tyrosine kinase growth factor receptors have been cloned into this retroviral vector. So far, EGFR, neu T, neu N, and erbB4 have been successfully cloned. Concentrated retroviral stocks have been prepared for each of these constructs. These viral stocks will be used in future experiments for infusing the mammary glands of non-transgenic and MMTV-neu N transgenic rats.

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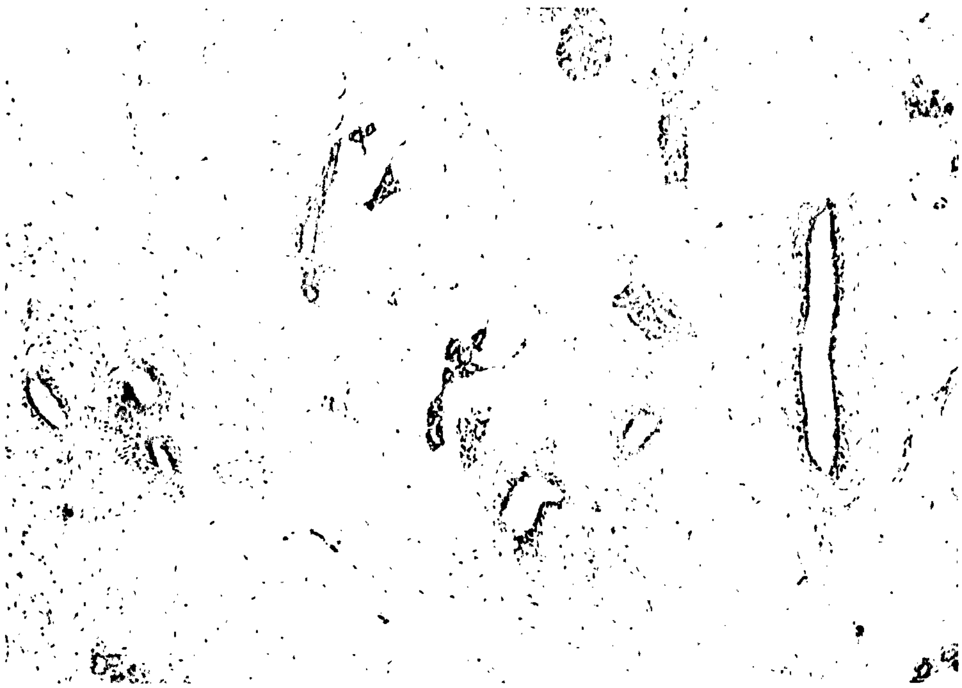
APPENDICES



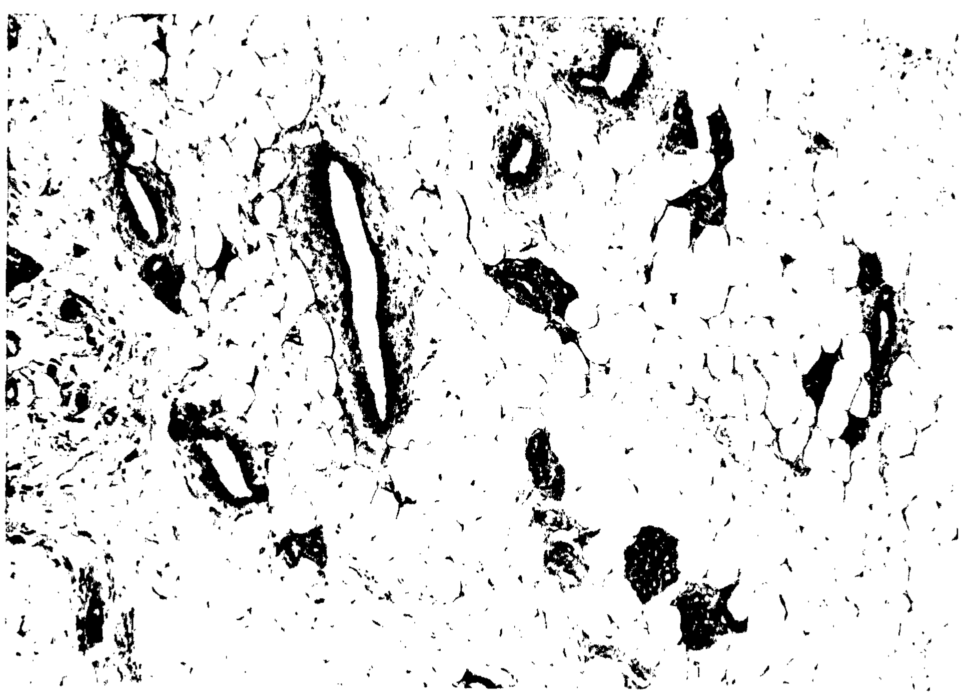
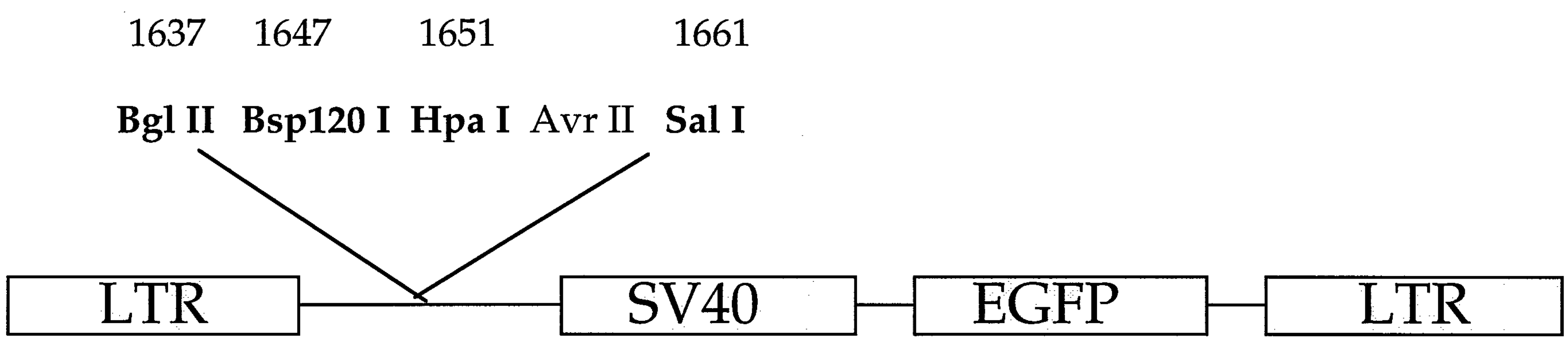


Figure 1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Avr II
Sal I
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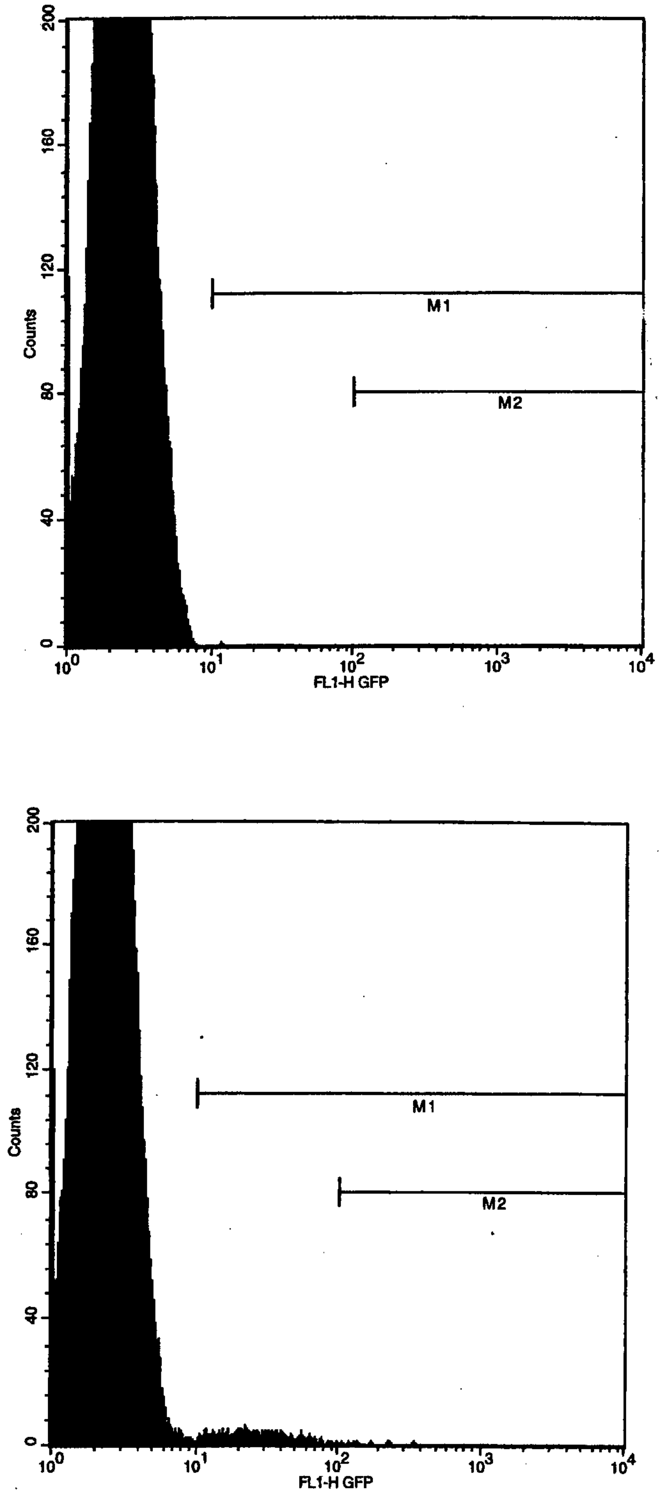
EGFP
LTR

Figure 2



Counts
0
40
80
120
160
200
M1
M2
10^0
10^1
10^2
10^3
10^4
FL1-H GFP
Counts
0
40
80
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M1
M2
10^0
10^1
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FL1-H GFP

Figure 3

FIGURE LEGENDS

Figure 1: Immunohistochemical Detection of neu in Rat Mammary Gland

Mammary gland tissue sections from a 3 month old non-transgenic rat were blotted with 0.5 µg/ml of either normal rabbit IgG (top panel) or rabbit anti-neu polyclonal IgG (bottom panel). Both sections were counterstained with hematoxylin. Photos are at 100X.

Figure 2: Retroviral Expression Vector pLSG

Schematic diagram of the vector pLSG. Long terminal repeat (LTR), simian virus 40 promoter (SV40), enhanced green fluorescent protein (EGFP). **Bold** indicates unique restriction sites. Note: figure not constructed to scale.

Figure 3: Flow Cytometry Assay for Calculation of LSG-based Retroviral Titers

NIH 3T3 were either mock-infected (top panel) or infected with a viral stock of LSG/neu T that had been diluted 10,000-fold (bottom panel). 50,000 infected cells were analyzed by flow cytometry and the results are represented as a histogram. The y-axis is number of cells and the x-axis is log scale GFP fluorescence. The area under M1 defines GFP^+ cells and was used for the titer calculations. M1 values are 0.00% (top panel) and 0.85% (bottom panel). The area under M2 is depicted simply to show the bright GFP^+ cell population.